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Title Page

CYP3A5 mRNA degradation by nonsense mediated mRNA decay (NMD)

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Running Title Page

Running title: degradation of CYP3A5 mRNAs.

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Abbreviations: PTC, premature termination codon; NMD, nonsense mediated decay; PTB, polypyrimidine tract binding protein; SNP, single nucleotide polymorphism.

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Abstract

The total CYP3A5 mRNA level is significantly greater in individuals carrying the *CYP3A5*1* allele than in *CYP3A5*3* homozygotes. Most of *CYP3A5*3* mRNA includes an intronic sequence (exon 3B) containing premature termination codons (PTC) between exons 3 and 4. Two models were used to investigate the degradation of CYP3A5 mRNA: a *CYP3A5* minigene consisting of *CYP3A5* exons and introns 3-6 transfected into MCF7 cells, and the endogenous *CYP3A5* gene expressed in HepG2 cells. The 3'-UTR g.31611C>T mutation has no effect on CYP3A5 mRNA decay. Splice variants containing exon 3B were more unstable than *wt* CYP3A5 mRNA. Cycloheximide prevents the recognition of PTCs by ribosomes: in transfected MCF7 and HepG2 cells, cycloheximide slowed down the degradation of exon 3B-containing splice variants, suggesting the participation of nonsense mediated decay (NMD). When PTCs were removed from pseudo-exon 3B or when UPF1 siRNA was used to impair the NMD mechanism, the decay of splice variant was reduced, confirming the involvement of NMD in the degradation of CYP3A5 splice variants. Induction could represent a source of variability for *CYP3A5* expression and could modify the proportion of splice variants. The extent of CYP3A5 induction was investigated after exposure to barbiturates or steroids: CYP3A4 was markedly induced in a pediatric population as compared to untreated neonates. However, no effect could be detected in either the total CYP3A5 RNA, the proportion of splice variant RNA or the protein level. Therefore, in these patients, induction is unlikely to switch on the phenotypic *CYP3A5* expression in *CYP3A5*3/*3* individuals.

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Introduction

The members of the CYP3A subfamily are responsible for the metabolism of more than 50 % of clinically used drugs (Eichelbaum and Burk, 2001), but the contribution of individual CYP3A isoforms is variable. The function of CYP3A43 has not yet been elucidated but its involvement in the overall drug metabolism should be of a minor importance as judged by its low mRNA level in different tissues (Domanski et al., 2001; Gellner et al., 2001). CYP3A7 is mostly expressed in the fetal liver and progressively replaced by CYP3A4 after birth (Lacroix et al., 1997). CYP3A4 and CYP3A5 are likely to play the major metabolic role in adult liver. CYP3A4 is constitutively expressed in the adult liver (Westlind-Johnsson et al., 2003), whereas CYP3A5 is polymorphically expressed at an appreciable level in only a fraction of ethnic populations (Hustert et al., 2001; Kuehl et al., 2001; Lin et al., 2002).

Several authors proposed that CYP3A5 could be only a minor contributor in the overall metabolism of CYP3A substrates (Fukuda et al., 2004; Shih and Huang, 2002; Westlind-Johnsson et al., 2003). However, at least for a small number of drugs, the CYP3A5 polymorphism plays a significant role in pharmacokinetics. Recently, a clear association between tacrolimus dose requirement and the CYP3A5 genotype has been described (Macphee et al., 2002; Zheng et al., 2003; Hesselink et al., 2003). A dose-dependent effect was also observed with ABT-773 (Katz et al., 2004). Therefore, it is likely that the pharmacokinetics of other CYP3A substrate drugs could be dependent, at least partially, on the CYP3A5 polymorphism (Huang et al., 2004).

The low level of CYP3A5 protein expression is linked to the *CYP3A5*3* allele. This allele displays a g.6986A>G (refSNP ID: rs776746) transition, creating a cryptic acceptor splice site in the third intron of the *CYP3A5* gene (numbering according to the recommendations of the CYP Allele Nomenclature Committee, Oscarson and Ingelman-Sundberg, 2002). This cryptic site promotes the insertion of an intronic exon-like sequence (pseudo-exon 3B), in the mature

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messenger RNA and could involve subsequent deletions of exon and/or insertion of other intronic sequences. These abnormally spliced messengers contain several in-frame premature termination codons (PTC), explaining the low protein level. In the past, several authors reported that *CYP3A5* is not inducible (Wrighton et al., 1989), but recently, Burk et al. (2004) have demonstrated a substantial increase in CYP3A5 mRNA levels in human hepatocytes after treatment with several known CYP3A inducers. The induction of CYP3A5 could therefore constitute a non-negligible source of inter-individual CYP3A5 mRNA and protein variability.

The objective of this work was to gain insight into the molecular regulation of the CYP3A5 mRNA level which is significantly lower in *CYP3A5*3/*3* individuals than in **1* carriers. No mechanism explaining this difference has been demonstrated so far even if several hypotheses have been proposed. First, we demonstrated that the g.31611C>T mutation (refSNP ID: rs15524) has no effect on the degradation of CYP3A5 mRNA. Then, we tested whether the lowest CYP3A5 mRNA level could result from the rapid degradation of splice variants by the nonsense mediated mRNA decay (NMD) and the role of PTC in this mechanism. Lastly, we investigated whether exposure to CYP3A inducers could modify the concentration and distribution of CYP3A5 mRNA in a panel of human livers. Since none of our adult samples were medicated we used samples obtained from neonates or newborns treated with either barbiturates or steroids.

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Materials and methods

Human liver bank. Caucasian neonatal and adult samples were obtained within the first hours following death in accordance with the recommendations of the Ethical Committee from Institut National de la Santé et de la Recherche Médicale. Neonatal livers were obtained after informed parental consent. The treatment of newborns with barbiturates or steroids was documented and allowed to classify the patients as "induced" as reported in Cresteil et al. (1994). Adult livers came from donors for kidney transplantation exempt of chronic diseases and received no medication known for their inducible capacity of CYP3As. Samples were immediately frozen in liquid nitrogen and then stored at -80°C prior to analysis.

Western-blotting of CYP3A5. Human liver microsomes were prepared according to a protocol reported previously (Cresteil et al., 1979). Microsomal protein content was determined using the Uptima BCA assay kit (Interchim, France). Sixty µg of microsomal proteins were resolved on 9% SDS-polyacrylamide gels and transferred overnight onto PVDF membranes (NEN, Boston, Ma). Membranes were incubated for 2 h with a polyclonal anti-human CYP3A5 antibody specific for CYP3A5 (Affiniti, Exeter, UK) or a polyclonal CYP3A4 antibody reacting with CYP3A4, 3A5 and 3A7 (Daiichi, Tokyo, Japan). Blots were revealed after 4-chloronaphtol staining. After scanning, the band intensities were measured using the Densylab densitometric software (Microvision, Evry, France). Results were normalized using the intensity of the band from the same sample loaded on each blot and expressed as optical density units per mg of microsomal proteins.

Genomic DNA preparation. Genomic DNA was purified from liver samples using the GenomicPrep™ Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Little

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Chalfont, UK) according to the manufacturer's instructions except for the rehydration step in which sterile water was used instead of the provided solution in order not to interfere with subsequent amplification steps. The genomic DNA samples were stored at 4°C.

Primer design, CYP3A5 *genotype determination*. Genbank accession number of sequences are: AC005020.5 for *CYP3A5*; AF280107.1 for *CYP3A4*, *CYP3AP2*, *CYP3A7*, *CYP3AP1*, and for *CYP3A43* promoter region; M18907.1, J04813.1, D00408.1, AF319634.1 for CYP3A4, CYP3A5, CYP3A7 and CYP3A43 mRNA respectively; and X03205.1 for 18S ribosomal RNA. Genomic and RNA sequences of CYP3A4, CYP3A5, CYP3A7, CYP3A43, *CYP3AP2* and *CYP3AP1* were aligned using the Multalin multiple sequence alignment software (Corpet, 1988). Oligos were selected using the primer3 PCR primer design program (Rozen and Skaletsky, 2000) and were designed to ensure selectivity toward CYP3A5 sequence. The sequence of oligos is shown in table 1. Oligos for genotyping the *CYP3A5*3* alleles (5020_22719, 5020_24161, 5020_22743, 5020_23205) were designed by Kuehl et al. and *CYP3A5*1/*3* genotyping was performed as described previously (Kuehl et al., 2001).

Plasmid construction. pMT2-3A5, a mammalian expression vector containing the *CYP3A5*1* cDNA was prepared previously (Santos et al., 2000). Oligos designed to hybridize to exons 2 and 8 were tailed with *Xho* I and *Kpn* I without modification of the open reading frame. They were used to amplify the pMT2 sequence flanked with exons 1-2 and 8-13 spliced CYP3A5 exons. Genomic DNA from a **3/*3* individual was amplified by high fidelity PCR from exon 3 to exon 7 with oligos tailed with *Xho* I-modified exon 2+3 and *Kpn* I-modified exon 7+8 sequences. The genomic insert and pMT2 flanked fragment were digested with *Xho* I and *Kpn* I, and ligated with T4 DNA ligase to obtain the p*3A5*3C* construct containing intronic and exonic sequences from exon 3 to exon 7 into *3A5*1* cDNA.

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The p*3A5*3C* minigene was entirely sequenced and presented the SNP configuration of the *CYP3A5*3C* allele (g.6986G and g.31611C). The structure of the minigene used herein is shown in figure 1. The GeneEditor™ in vitro Site-Directed Mutagenesis System (Promega) was used to generate modified constructs. p*3A5*3C*-ΔPTC was obtained after removal of the 4 premature terminaison codons present in exon 3B with oligo: TGT GTC ACA CCC **A**AA **C**GA ACT AGA ACC **A**AA GGT TGC TGT GTG TCG TAC AAC **A**AG GGG TAT GGA TTA C. p*3A5*3A* is obtained after mutation of p*3A5*3C* with oligo ATT CTA AGG A**T**T TCT ACT TTG and reproduces the *CYP3A5*3A*-like sequence (g.6986G and g.31611T).

Cell culture. Human breast carcinoma MCF7 and human hepatocellular carcinoma HepG2 cells were grown at 37°C under 5% CO_2, respectively, in DMEM and MEM media supplemented with 10 % fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1.5 µg/ml amphotericin B (Invitrogen, Cergy-Pontoise, France).

Cell transfections. For the measurement of RNA decay, 5.10^5 MCF7 cells were seeded in 6-well plates. After 24 hours, cells were transfected, using the calcium phosphate method with 5 µg of minigene construct (p*3A5*3C* or p*3A5*3C*-ΔPTC) and 2 µg of pRL-SV40 (Promega). On day 3, transfected cells were incubated with 5 µg/ml actinomycin-D (Acros Organics, Geel, Belgium) for 2, 4, 6 and 8 hours before RNA isolation with or without pretreatment with 20 µg/ml cycloheximide for 2 hours (Acros Organics).

The effect of cycloheximide (20 µg/ml) on CYP3A5 mRNA level after mock, p*3A5*3C*, p*3A5*3C*-ΔPTC or p*3A5*3A* transfection, was investigated in MCF7 cells after an incubation of 8 h using the same protocol, except that cells were not treated by actinomycin-D. The effect of cycloheximide (20 µg/ml) on endogenous CYP3A5 mRNA level was also

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investigated in native HepG2 cells. Total RNA was extracted from frozen liver samples or cultured cells using the Promega SV Total RNA Isolation System.

siRNA experiments. For siRNA experiments, MCF7 and HepG2 cells were seeded in 12-well plates and transfected on day 2 with 60 pmoles of GL2 or UPF1 siRNAs using 3 µl of lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Annealed siRNA duplexes were purchased from MWG biotech (Ebersberg, Germany); mRNA targets for gene-specific knockdown were: AAC GUA CGC GGA AUA CUU CGA for the firefly luciferase gene (Elbashir et al., 2001) and: AAG AUG CAG UUC CGC UCC AUU for the human UPF1 (RENT1) protein (Mendell et al., 2002). On day 3, cells were trypsinized and split at a low density into fresh 12-well plates, and transfection with 60 pmoles of siRNA were repeated on day 6 using 3 µl of oligofectamine (Invitrogen). Cells were trypsinized on day 7 and split in new 12-well plates before be transfected on day 8 with 100 ng pGL2-pm (Promega), 50 ng pRL and 500 ng of either minigene constructs with 3 µl of lipofectamine 2000. Cells were harvested on day 9 and RNA extracted with Trizol followed by digestion with RQ1 DNase (Promega) according to the manufacturer's recommendations.

The luminescence emitted by the firefly luciferase synthesized from pGL2 and pRL was measured using the Dual-Glo™ Luciferase Assay System (Promega) on a Berthold Lumat LB 9507 luminometer.

The integrity of RNA was checked by electrophoresis in 1% agarose gels in 1X TAE buffer. One µg of total RNA were reverse transcribed using 500 ng of random hexamers and 200 units MMLV Reverse Transcriptase (Promega) in a 25 µl final volume according to the manufacturer protocol.

PCR, quantitative Real-Time PCR and data normalization. Total RNA was reverse transcribed and diluted 1:40 with PCR grade water for the detection of CYP3A5 and GAPDH

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cDNA or with an additional $1:10^4$ dilution for 18S rRNA cDNA detection. Quantitative real-time PCR experiments were performed with Lightcycler FastStart DNA Master Plus SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany), with oligos at a 300 nM final concentration in glass-capillaries in a 10 µl final volume. Total CYP3A5 mRNA was measured using oligos 2F and 3R, while splice variants (*SV*) which contain the pseudo-exon 3B were amplified using oligos MF and MR. CYP3A5 mRNAs correctly spliced at exon-junctions 3-4 and 6-7, referred to as wild-type CYP3A5 mRNA (*wt*), were amplified using OKF and OKR. PTB mRNA were amplified by PCR with oligos 2132 and D3'2 described by Wollerton et al. (2004). These oligos allowed the amplification of a product spanning exons 10 to 12 and the detection of wild-type mRNA and splice variants lacking exon 11.

Hepatic CYP3A5 mRNA level was normalized with 18S rRNA, a suitable housekeeping gene for human liver samples (Koch et al., 2002). RNA decay experiments were normalized with 18S rRNA which is transcribed with an actinomycin-D insensitive polymerase. Q-PCR normalization was performed with the Renilla luciferase mRNA level in transiently transfected MCF7 and with endogenous GAPDH mRNA level for experiments performed in HepG2 cells. In siRNA experiments, to allow comparison between cell lines which present different levels of CYP3A5 expression, we expressed results as the ratio between 3B splice variants and *wt* CYP3A5 mRNA levels.

PCR were performed with the PCR Core System I (Promega) with oligos 2F and 4R for the simultaneous amplification of *wt* and 3B variants. PCR products were analyzed by electrophoresis in 2.5 % agarose gels.

Data Analysis. Statistical significance was assessed by Mann-Whitney U test or Student t test using a spreadsheet program.

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Results

1) Quantification of CYP3A5 mRNA in human livers

Twenty seven samples were genotyped for the *CYP3A5*3* allele and the CYP3A5 mRNA levels was determined by quantitative real time PCR (Fig. 2). It is noteworthy that in all samples bearing at least one CYP3A5*3 allele, the total RNA level is a composite figure of correctly spliced and splice variants in variable amounts. The CYP3A5 mRNA level is expressed in arbitrary units, with a value set to 1 for our single homozygous **1/*1* liver. Livers from non-induced individuals were divided into subpopulations according to their genotype: *CYP3A5*3/*3* (n=14), *CYP3A5*1/*3* (n=7) and *CYP3A5*1/*1* (n=1) individuals. The level of total CYP3A5 mRNA in the whole population varied over a 53-fold range. This variability fell to 9 (range: 0.050-0.439) and 5 (range: 0.539-2.651) within *CYP3A5*3/*3* and **1/*3* subpopulations respectively. Ranges were not overlapping and the difference between subgroups was statistically significant (Mann-Whitney U test, $p<0.001$). When the proportion of correctly spliced CYP3A5 mRNA was examined, a significantly higher amount was observed (Mann-Whitney U test, $p<0.001$) in *CYP3A5*1/*3* (range: 0.643-0.883) than in **3/*3* individuals (range: 0.086-0.358), respectively with a 1.5- and 4-fold intra-group variability. These results are consistent with the study of Lin et al. (2002), although the ratio of correctly spliced mRNA / splice variants mRNA is different in **3/*3* individuals.

Interestingly, the total CYP3A5 mRNA level is greater in **1* carriers than in **3/*3* homozygotes. This information suggests either a difference in CYP3A5 mRNA stability or a reduction in the transcriptional activity of *CYP3A5*3*. So far, no SNP affecting the 5' regulatory region of the *CYP3A5* gene has been reported to be in linkage disequilibrium with the *CYP3A5*3* mutation, but this latter explanation cannot be excluded. Therefore, we wished

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to determine whether the mRNA stability could be modified in CYP3A5 splice variants that incorporate pseudo-exon 3B.

2) CYP3A5 wild-type and splice variant decay

We developed two cellular models to explore the mechanism underlying CYP3A5 mRNA decay. First, the *CYP3A5* genotype and mRNA expression level were investigated in the MCF7 human breast carcinoma and the HepG2 human hepatocellular carcinoma cell lines. These two cell lines have the *CYP3A5*3/*3* genotype. MCF7 cells present a very low basal CYP3A5 mRNA accumulation and constitute a suitable model to investigate the splicing and stability of CYP3A5 mRNA with an exogenous transfected CYP3A5 minigene construct. Conversely, HepG2 cells express CYP3A5 mRNA at a higher basal level than MCF7 cells (> 30-fold), allowing us to explore the splicing of the endogenous CYP3A5 gene (fig. 3).

MCF7 cells were transfected with p*3A5*3C* in order to investigate the stability of *wt* and splice variants by quantitative real-time PCR after treatment with actinomycin-D, an inhibitor of RNA synthesis. Data indicated that splice variants containing the pseudo-exon 3B decayed faster than the wild-type transcript (the half life of *wt* mRNA is 1.7-fold longer than that of splice variants), suggesting that the difference in the total CYP3A5 mRNA level between **1* carriers and *3/*3 individuals might be due to a different stability of RNA species (fig. 4).

Lee et al. (2003) suggested that the g.31611C>T mutation which is in linkage disequilibrium with the g.6986A>G SNP could be involved in the degradation of the CYP3A5 mRNAs. To evaluate the impact of this mutation on the decay of CYP3A5 mRNA in the

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MCF7 model, the level of CYP3A5 mRNA transcribed from p*3A5*3A* (generated by g.31611C>T site-directed mutagenesis of p*3A5*3C*) was compared to those produced by p*3A5*3C* 24 hours after transfection with either construct (Fig. 5). Neither wild-type nor splice variant CYP3A5 mRNA levels are modified by this SNP. Consequently the total CYP3A5 mRNA level was unaffected, which would indicate that the g.31611C>T transition is unlikely to be implicated in the degradation of CYP3A5 mRNAs. Consequently, we used in subsequent experiments the p*3A5*3C* minigene to carry out transfection in MCF7 cells and investigate the mechanism of splice variant degradation.

3) Protein synthesis inhibition experiments

A possible mechanism for the degradation of mRNAs containing premature termination codons (PTC) is called nonsense mediated mRNA decay (NMD) and involves ribosomes. Protein synthesis inhibitors have been widely used to demonstrate the role of NMD in the degradation of nonsense transcripts (Sureau et al., 2001; Harries et al., 2004; Lamba et al., 2003; Lei et al., 2001). Mock-transfected or MCF7 cells transfected with p*3A5*3C* were incubated or not with cycloheximide (fig. 6). The level of wild type and splice variant CYP3A5 mRNA was more than 30-fold greater in minigene transfected-MCF7 than in native MCF7 cells (Student's t test, $p<0.001$). Protein synthesis inhibition by cycloheximide resulted in an increase in the level of splice variant that contain the pseudo-exon 3B (Student's t test, $p<0.001$) (fig. 6a). This effect was not due to an induction of the endogenous *CYP3A5* gene as seen with mock-transfected MCF7 cells treated with cycloheximide. The treatment with cycloheximide has no significant effect on the amount of correctly spliced CYP3A5 mRNA, indicating that the effect might be specific to RNA molecules containing PTC (fig. 6b). Furthermore, cycloheximide slows the decay of splice variant RNA, but has no

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effect on the decay of correctly spliced mRNA (data not shown). To confirm the effect of cycloheximide on the decay of splice variant, PCR with different pairs of oligos were carried out with endogenously expressed *CYP3A5* in HepG2 cells. With oligos 2F and 4R, 253 bp and 121 bp were amplified with splice variant and wild-type RNA respectively (Fig. 7a). As shown in figure 7b, the addition of cycloheximide has no effect on the level of wild-type RNA whereas the level of splice variants is 2.5-fold higher than in cells treated with vehicle only. This was confirmed in amplification with oligos MF and MR, which assays the 3B sequence inserted in RNA. Thus cycloheximide elicits the same effect on the endogenous *CYP3A5* gene as in the transfected *CYP3A5*3C* minigene in MCF7, corresponding to a marked reduction of the decay of incorrectly spliced RNA, presumably through an inhibition of NMD.

4) Role of PTC in RNA stability

NMD is a protection mechanism that promotes the degradation of mRNA containing PTC. The pseudo-exon 3B is 132 bp long, and its insertion into the RNA molecule does not shift the ORF. Thus, mutation of PTC into amino acid coding sequences without modification of the g.6982A>G mutation should permit the synthesis of a splice variant RNA not degraded by NMD. The site-directed mutagenesis of p*3A5*3C* was designed as p*3A5*3C*-ΔPTC and transfected into MCF7 cells. The amount of CYP3A5 splice variant was compared after transfection of p*3A5*3C* containing the sequence 3B and its four PTC or p*3A5*3C*-ΔPTC containing the sequence 3B devoid of PTC.

A marked increase (x 3.8) in the level of splice variants containing pseudo-exon 3B was detected with p*3A5*3C*-ΔPTC (Fig. 8, $p<0.001$), and was associated with a reduction of the decay of the transcript when compared with splice variants produced from p*3A5*3C*

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(Fig. 4). This clearly indicates that the decay of splice variants is associated with the presence of PTCs, and suggests the participation of NMD in the degradation of *CYP3A5*3* splice variants.

5) Inhibition of hUPF1 by siRNA

NMD in mammalian cells depends on hUPF1 (or RENT1), a RNA-binding protein whose activity is modulated by ATP. Herein we have designed experiments to selectively knockdown hUPF1 with a siRNA described previously (Mendell et al., 2002). Crucial points are the use of adequate controls. To test the transfection efficiency with siRNAs, MCF7 cells were transfected with GL2 siRNA directed against the renilla luciferase mRNA: this resulted in an 85 ± 5 % (mean ± SEM) inhibition of the luciferase activity whereas transfection with UPF1 siRNA has no effect on the luciferase activity. To test the transfection with siRNA for hUPF1 in MCF7 or HepG2 cells, cellular RNA was extracted and used as template in RT-PCR for the amplification of the polypyrimidine tract binding protein (PTB) mRNA. A 30 ± 3 % (mean ± SEM) increase in PTB splice variant lacking exon 11, a known substrate of NMD (Wollerton et al., 2004), was noticed, indicating that NMD activity was reduced. Mock transfected cells and cells transfected with GL2 siRNA showed no variation in the level of PTB splice variant. Thus, hUPF1 siRNA acts specifically on the NMD activity.

The level of endogenous CYP3A5 splice variant mRNA is twice as high in HepG2 cells transfected with UPF1 siRNA as in mock transfected or in HepG2 cells transfected with GL2 control siRNA (Fig. 9a). Similar results were obtained in MCF7 cells transfected with exogenous *CYP3A5*3C* plasmid in the absence or presence of hUPF1 siRNA (Fig. 9b).

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These results confirm the involvement of NMD in the degradation of CYP3A5*3 splice variant containing the pseudo-exon 3B and its four PTC, and explain the rapid decay of *CYP3A5*3* RNA.

6) Inducibility of CYP3A5

It has been recently shown that addition of CYP3A inducers to human hepatocytes could substantially increase the CYP3A5 mRNA level (Burk et al., 2004). We wished to determine whether administration of barbiturates or steroids to patients at pharmacological doses could modify the splice variant/wild-type RNA ratio, shifting the **3/*3* genotype to a **1/*3* like phenotype. None of our adult samples were medicated and so did not allow this type of comparison. However, in our pediatric liver bank, five samples were obtained from neonates or newborns treated with barbiturates or steroids.

When compared with untreated samples in the same age group, samples treated with barbiturates displayed a higher CYP3A content associated with an enhanced testosterone-6β hydroxylation (table 2). In a child treated for 1 year with prednisone, no increase was noticed. When tested with an antibody specific for CYP3A5, only N77 was positive but his CYP3A5 mRNA content was comparable to that of age-matched newborns receiving no treatment. The four other samples had very low levels of CYP3A5 corresponding to a **3/*3* phenotype. The level of CYP3A5 mRNA and the proportion of wild-type CYP3A5 mRNA were investigated in these samples. Basically, the total CYP3A5 mRNA was comparable in pediatric and adult populations (fig. 2). When classified according to their genotype, **3/*3* neonates exhibited CYP3A5 RNA level close to that of **3/*3* adults; **1/*3* neonates were close to **1/*3* adults, and the proportion of correctly spliced RNA was comparable in pediatric and adult populations according to their genotype. Thus no age dependent effect could be demonstrated

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with these samples. When “induced” samples were examined, the values obtained with the four **3/*3* pediatric samples did not differ from those obtained with untreated **3/*3* children or adults (fig. 2). Their total RNA content and their proportion of correctly spliced RNA were similar to that of untreated age-matched individuals. N77, with a **1/*3* genotype and induced by phenobarbital, displayed an unexpectedly low CYP3A5 mRNA content and a depressed proportion of wild-type correctly spliced mRNA in the range of **3/*3* individuals. Consequently, our results do not support a potent CYP3A5 induction in five pediatric patients treated with barbiturates or steroids, although their total CYP3A content was markedly increased. Furthermore, treatment with barbiturate did not modify the proportion of splice variants mRNA nor the apparent decay of differently spliced RNA.

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Discussion

The CYP3A5 protein is found at an appreciable level in only 10-20 % of the Caucasian population. In the last decade, several hypotheses have been formulated to explain the polymorphism in CYP3A5 expression: (i) an instability of the newly synthesized protein, (ii) a reduced transcriptional level due to mutations in the promoter region, (iii) an altered level of mature RNA coding for the full length protein.

First, Jounaidi et al. (1996) proposed that a SNP (*CYP3A5*2* allele) could make the CYP3A5 protein unstable. However, the very low frequency of this allele (0.019, Hustert et al., 2001) could not explain the low level of protein in 80 % of the Caucasian population. Moreover, recombinant T398N mutant protein is detected by western blotting in microsomes from cells transfected with the *CYP3A5*2* cDNA at the same level than after transfection with wild type CYP3A5 cDNA and therefore does not appear to be unstable (unpublished data). Later, Paulussen et al. (2000) were concerned with an alteration of the transcriptional level of CYP3A5 and reported a good correlation between the CYP3A5 expression and the presence of two mutations in what was thought to be the *CYP3A5* promoter region and was in fact located upstream the *CYP3AP1* pseudogene. To date, no SNP correlated with CYP3A5 expression has been described in the promoter region of *CYP3A5* and no evidence has been provided showing a reduced transcription of CYP3A5 in a fraction of the population.

Kuehl et al. (2001) produced convincing evidence that the low level of CYP3A5 protein was associated with the presence of two *CYP3A5*3* alleles. The g.6986A>G mutation produced an alteration of the CYP3A5 splicing with the insertion of pseudo-exon 3B. This mutation resulted in a lower RNA content and was tentatively explained by a rapid degradation of splice variant by NMD. NMD is a protective process for the cell promoting degradation of mRNAs containing premature termination codons located more than 50

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nucleotides before the last exon-exon junction (Holbrook et al., 2004). With four PTC, splice variants of *CYP3A5*3* containing the pseudoexon 3B are possible substrates for NMD. However this hypothesis has not been verified so far. The last hypothesis proposed by Lee et al. (2003) implicated the g.31611C>T mutation. This mutation is in linkage disequilibrium with the g.6986A>G SNP. The g.31611C>T SNP creates an AUUUC motif in the 3' untranslated region (3'-UTR) of CYP3A5 mRNAs produced from *CYP3A5*3* alleles. This sequence resembles the AUUUA 3'-UTR destabilizing motif observed in some highly regulated mRNAs (Wilusz et al., 2001).

The goal of the present study was to gain insight into the molecular mechanism controlling CYP3A5 expression. For that purpose, two models were designed to test results. A "minigene" was built containing introns 3-6 to generate splice variants. This construction was transfected into MCF7 cells, a cell line having an extremely low basal expression of the endogenous *CYP3A5* gene. Data obtained after transfection experiments were compared with those of the endogenous *CYP3A5* expression in HepG2, a cell line with an active *CYP3A5*3* gene transcription, generating splice variants. The initial observation was related to the decay of CYP3A5 mRNA in cell lines: splice variants which incorporate the pseudo-exon 3B are less stable than wild type mRNA: this clearly indicates that in individuals bearing the **3/*3* genotype, the lowest CYP3A5 mRNA level could result from a rapid degradation of splice variants produced by *CYP3A5*3*.

In a first attempt, to tentatively explain the degradation of splice variants RNA, the role of the g.31611C>T mutation was investigated. Reversal of this mutation to the *wt* genotype has no effect on the decay of splice variants containing the g.6986A>G mutation. This clearly indicates that the mutation in the 3' UTR does not cause the faster degradation of the splice variant RNA. Alternatively, the presence of multiple premature stop codons could suggest the implication of NMD in the RNA degradation. To test this hypothesis, cells were

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treated with cycloheximide, a molecule known to prevent protein synthesis and indirectly the NMD RNA degradation. Addition of cycloheximide resulted in a marked accumulation of splice variant through an inhibition of its fast degradation but has no effect on the degradation of the *wt* RNA. Therefore, a functional translation machinery is required for the degradation of splice variants RNA. The removal of PTC from splice variant significantly reduced the degradation of splice variant indicating the role of PTC in the degradation process through an activation of NMD. Lastly, the silencing of hUPF1 by siRNA downregulated the NMD pathway and was accompanied by a reduction of splice variant degradation.

Altogether, these experiments conducted both with the exogenous minigene transfected in MCF7 cells and the endogenous *CYP3A5* gene expressed in HepG2 cells strongly suggested the participation of NMD in the degradation of splice variant whereas the *wt* CYP3A5 RNA is not affected.

In agreement with our *in vitro* cellular models, published results obtained *in vivo* from liver samples support the existence of an allele specific degradation of CYP3A5 mRNA. In heterozygous g.31611C/T samples, only cDNA derived from the sequence containing the cytosine was detected and is called pseudohomozygocity by Wojnowski and Brockmoller (2004). The authors attribute this over representation to the tight linkage of g.31611C with g.6986A. These observations could be interpreted as a lower accumulation of *CYP3A5*3* RNA than *CYP3A5*1* RNA consequent to the rapid degradation of *CYP3A5*3* mRNA containing PTC by NMD in agreement with our observations.

Recently, Burk et al. (2004) described a PXR-dependent induction of CYP3A5 which could phenocopy the effect of the high expression allele **1* in individuals bearing the **3/*3* genotype. It could be speculated that the overall mRNA synthesis is stimulated, keeping

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constant the proportion of correctly and incorrectly spliced molecules synthesized. Since the splice variant is more rapidly degraded than the correctly spliced molecule, this should result in a higher content of *wt* RNA molecule and consequently to a higher protein synthesis. However, in our hands, no such observations have been validated: in **3/*3* genotyped samples, the proportion of splice variant / wild-type mRNA remains constant regardless of the induction by phenobarbital or prednisone and no accumulation of CYP3A5 protein was noticed in contrast with the increased CYP3A (presumably 3A4) and related activity testosterone-6β-hydroxylase. These results have been obtained in pediatric samples treated with therapeutic doses of phenobarbital. In our samples the expression of CYP3A5 in the Caucasian population was conserved in neonatal and adult group with about 15% of positive CYP3A5 samples, and the ontogenic pattern of CYP3A5 follows that described for CYP3A4 in the same liver bank (unpublished data). Stevens et al. (2003) also detected immunoreactive CYP3A5 protein in postnatal samples. Although the number of pretreated human samples was very limited, our data do not support the existence of a significant induction of hepatic *CYP3A5* at this stage of development. Presently, we cannot provide convincing evidence to explain the failure of phenobarbital to induce CYP3A5.

In conclusion, we confirm a previously published observation reporting a lower level of CYP3A5 mRNA in *CYP3A5*3/*3* individuals as compared to *CYP3A5*1* carriers. The splice variants generated by *CYP3A5*3* allele contained multiple premature stop codons and are degraded by nonsense mediated mRNA decay faster than the wild type mRNA, which explains the difference in mRNA level observed between the two genotype subgroups. In pediatric samples obtained from barbiturate-treated children, the level of total CYP3A5 RNA and the proportion of splice variant mRNA were not modified, and correlated with the low level of CYP3A5 protein.

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Legends for Figures

Figure 1: Structure of p3A5*3 *minigenes*.

Three CYP3A5 minigenes were designed to include all CYP3A5 exons and introns between exons 3 and 7. The nucleotide bases at g.6986 and g.31611 positions as well as the number of premature termination codons (PTC) are indicated for each minigene construct. Exons are depicted by white boxes and grey boxes represent intronic pseudo-exons.

Figure 2: CYP3A5 mRNA level in human livers.

(a) The total CYP3A5 mRNA content and proportion of wild-type (*wt*) mRNA (set to 1 for our single **1/*1* individual), measured by quantitative real-time PCR, are indicated for each individual enrolled in our study. Adult samples are depicted by circles; squares and triangles represent untreated children and children having received known CYP3A inducers. Data point colors indicate the genotype of individuals: white, grey and black for **3/*3*, **1/*3* and **1/*1* individuals respectively. Panel (b) is an enlargement of the doted area in panel (a).

Figure 3: CYP3A5 mRNA levels observed in HepG2 and MCF7 cell lines and in human hepatic samples.

The CYP3A5 mRNA content, measured by quantitative real-time PCR, is expressed in arbitrary units (set to 1 for the **1/*1* individual). Error bars indicate the standard error of the mean. An enlargement of the graph is provided for HepG2 and MCF7 cell lines.

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*Figure 4: CYP3A5 mRNA decay in MCF7 cells transfected with p*3A5*3 *constructs*.

Twenty four hours after transfection with CYP3A5 minigenes, MCF7 cells were incubated with actinomycin-D and the level of wild-type (*wt*) or splice variant (*SV*) CYP3A5 mRNA were measured by quantitative real time PCR at different times.

Figure 5: Effect of the 3' untranslated region g.31611C>T transition on CYP3A5 mRNA level.

The levels of splice variant (*SV*, panel a), wild-type (*wt*, panel b) and total (c) CYP3A5 mRNA were quantified by quantitative real time PCR in MCF7 cells 24 hours after transfection with either p*3A5*3C* or p*3A5*3A* constructs. mRNA levels are expressed in arbitrary units set to 1 for p*3A5*3C* transfected cells. Error bars indicate the standard error of the mean.

Figure 6: Effect of cycloheximide treatment on CYP3A5 mRNA level in transfected MCF7 cells.

The levels of splice variant (*SV*, panel a) and wild-type (*wt*, panel b) CYP3A5 mRNA were quantified by quantitative real time PCR in MCF7 cells transfected with p*3A5*3C* or mock transfected and treated or not with cycloheximide (CHX) for 8 hours. mRNA levels are expressed in arbitrary units set to 1 for untreated p*3A5*3C* transfected cells. Error bars indicate the standard error of the mean.

Figure 7: Effect of cycloheximide treatment on CYP3A5 mRNA level in HepG2 cells.

The levels of endogenous splice variant (*SV*, panel a) and wild-type (*wt*, panel b) CYP3A5 mRNA were quantified by quantitative real time PCR in HepG2 cells treated or not with

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cycloheximide (CHX) for 8 hours. mRNA levels are expressed in arbitrary units set to 1 for untreated HepG2 cells. Error bars indicate the standard error of the mean.

Figure 8: Effect of premature termination codon removal on CYP3A5 splice variant mRNA level in MCF7 cells.

CYP3A5 splice variant mRNA level was quantified by quantitative real time PCR in MCF7 cells transfected with p*3A5*3C* and p*3A5*3C*-ΔPTC. Splice variant mRNA levels are expressed in arbitrary units set to 1 for p*3A5*3C* transfected cells. Error bars indicate the standard error of the mean.

Figure 9: Effect of siRNA transfection on CYP3A5 mRNA level.

(a) CYP3A5 splice variant (*SV*) and GAPDH mRNA from HepG2 cells transfected with UPF1 or GL2 siRNA were amplified by PCR and electrophoresed on agarose gels. (b) CYP3A5 splice variant / wild-type mRNA level were measured by quantitative real-time PCR in HepG2 and in p*3A5*3C* transfected MCF7 cells. The cells were either mock, UPF1 or GL2 siRNA transfected, and the ratio was arbitrarily set to one for mock transfected cells. Error bars indicate the standard error of the mean.

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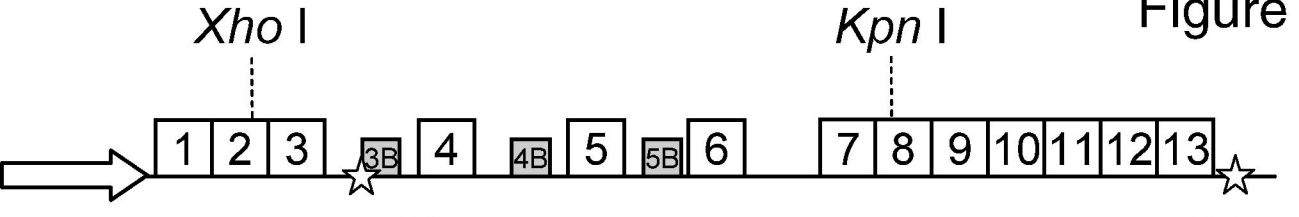
Table 1:

Oligo	Sequence
2F	ccc aca cct ctg cct ttg
3R	cca cat ttt tcc ata ctt ttt ata gca c
4R	cag gga gtt gac ctt cat acg
MF	ggg gta tct ctt ccc tgt ttg
MR	gac ctt cat acg ttc cct agt tg
OKF	aag tat gga aaa atg tgg gga ac
OKR	ctg tag gcc cca aag atg tc
18SF	ctg aga aac ggc tac cac atc
18SR	cgc tcc caa gat cca act ac
GAPDHF	ccc ttc att gac ctc aac tac at
GAPDHR	acg ata cca aag ttg tca tgg at
RL-F	agg cac tgg gca ggt gt
RL-R	cat ccg ttt cct ttg ttc tgg

Table 2:

Sample	Age	Treatment (length)	Total CYP3A protein uDO.mg protein^{-1}	CYP3A5 protein uDO.mg protein^{-1}	Testosterone-6β hydroxylase nmoles.min^{-1}.mg protein^{-1}
			(% of untreated samples in the same group of age)		
86	6 days	PB (4 days)	1.28 (228 %)	0	0.281 (1873 %)
49	16 days	PB (9 days)	1.06 (171 %)	0.015	0.053 (139 %)
77	3 months	PB (3 days)	1.44 (212 %)	0.426	0.208 (371 %)
79	9 years	Prednisone (1 year)	0.45 (79 %)	0	0.066 (55 %)
83	9 years	PB (7 days)	1.38 (242 %)	0.009	0.305 (252 %)

Figure 1



Minigene	Number of PTC in pseudo-exon 3B		
p*3A5*3A*	G	4	T
p*3A5*3C*	G	4	C
p*3A5*3C*-ΔPTC	G	0	C

Figure 2

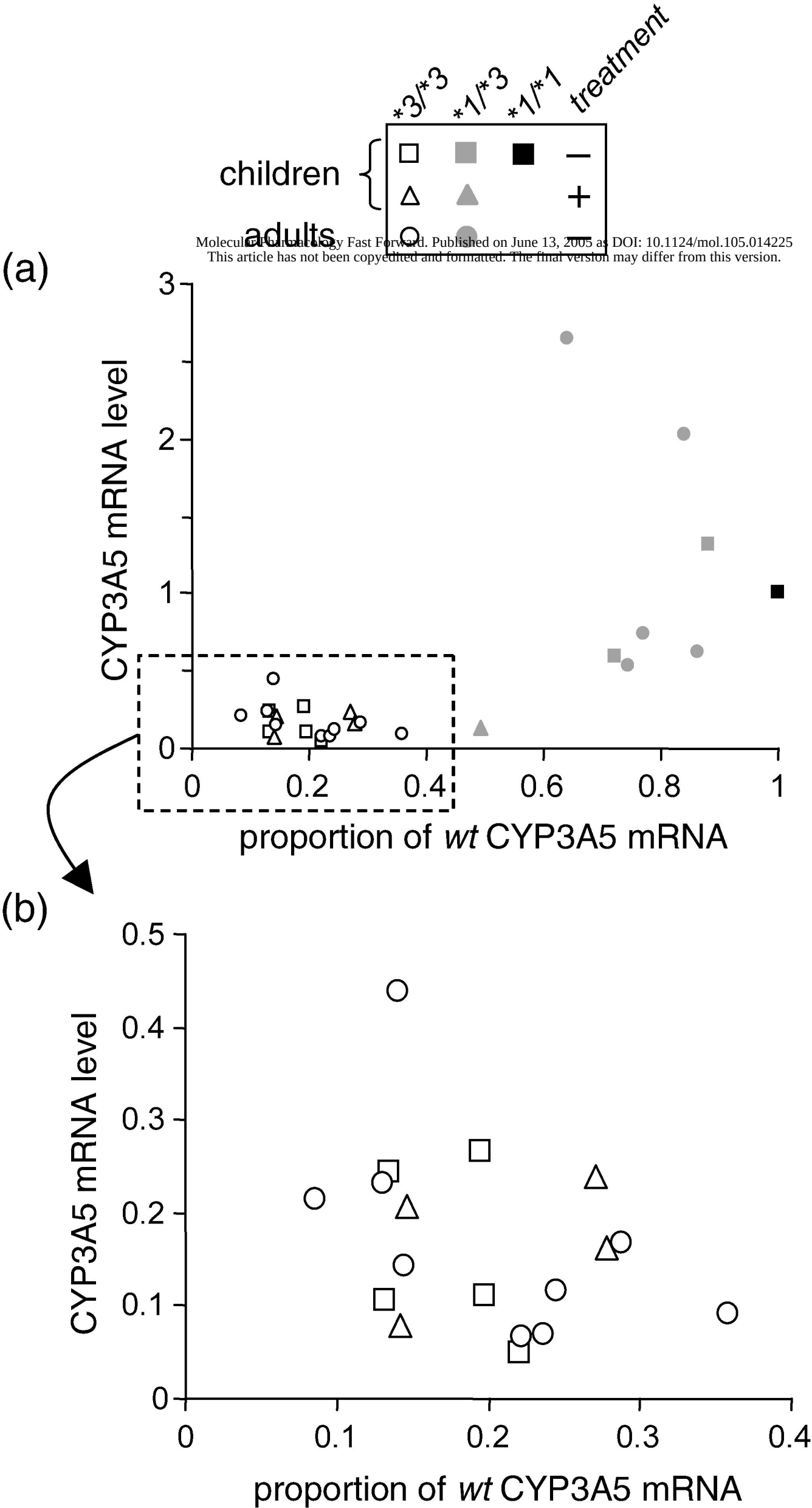


Figure 3

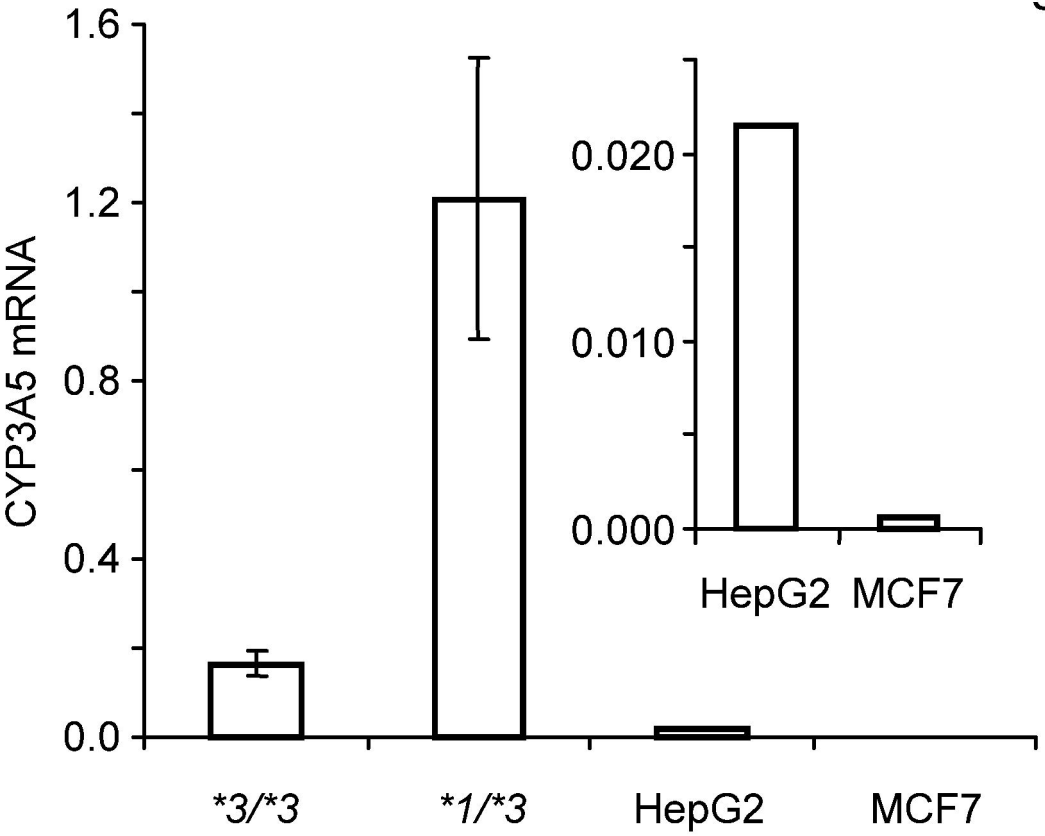

1.6
1.2
0.8
0.4
0.0
CYP3A5 mRNA
0.020
0.010
0.000
HepG2
MCF7
*3/*3
*1/*3
HepG2
MCF7

Figure 4

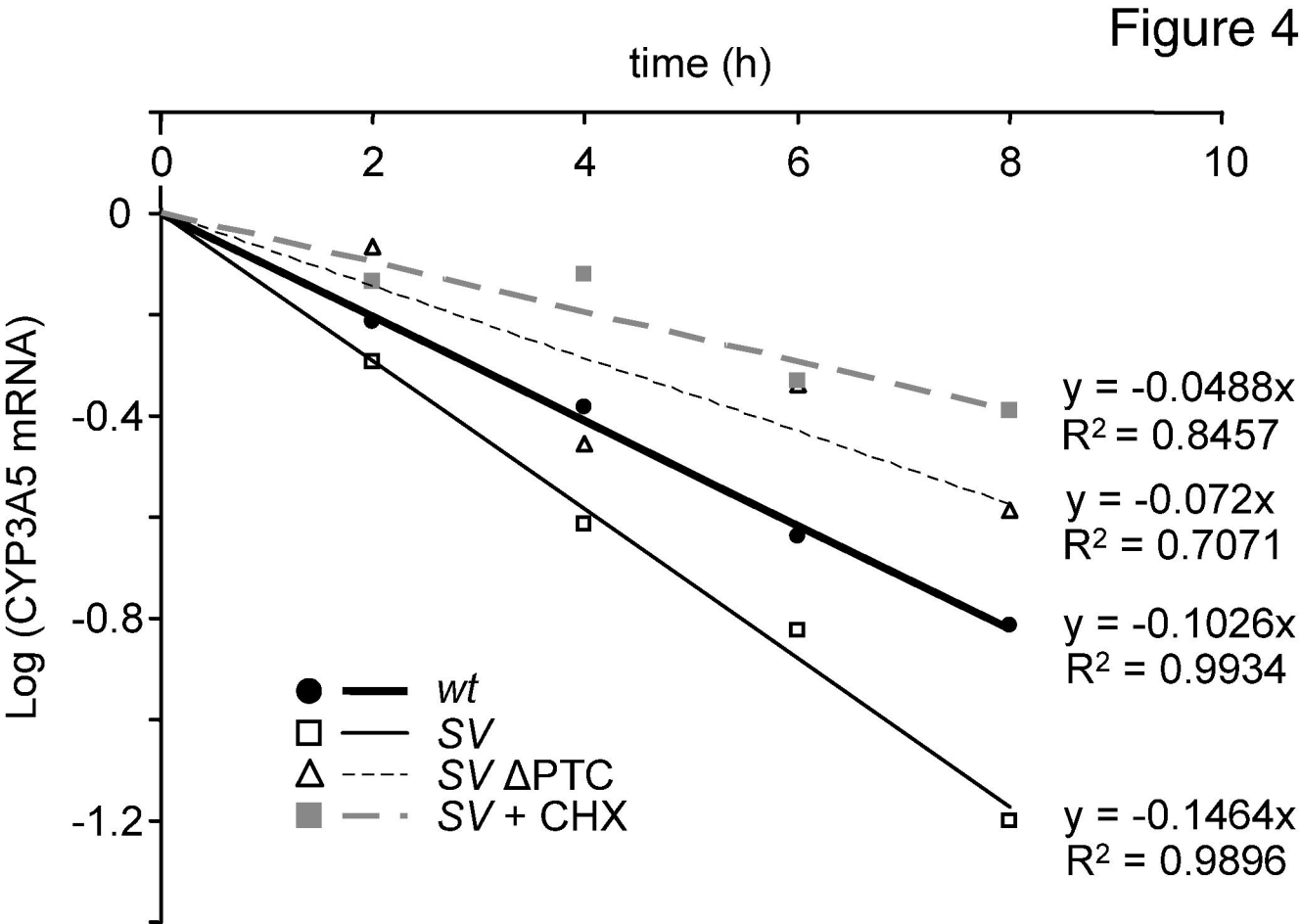

time (h)
0
2
4
6
8
10
0
-0.4
-0.8
-1.2
Log (CYP3A5 mRNA)
wt
SV
SV ΔPTC
SV + CHX
y = -0.0488x
R2 = 0.8457
y = -0.072x
R2 = 0.7071
y = -0.1026x
R2 = 0.9934
y = -0.1464x
R2 = 0.9896

Figure 5

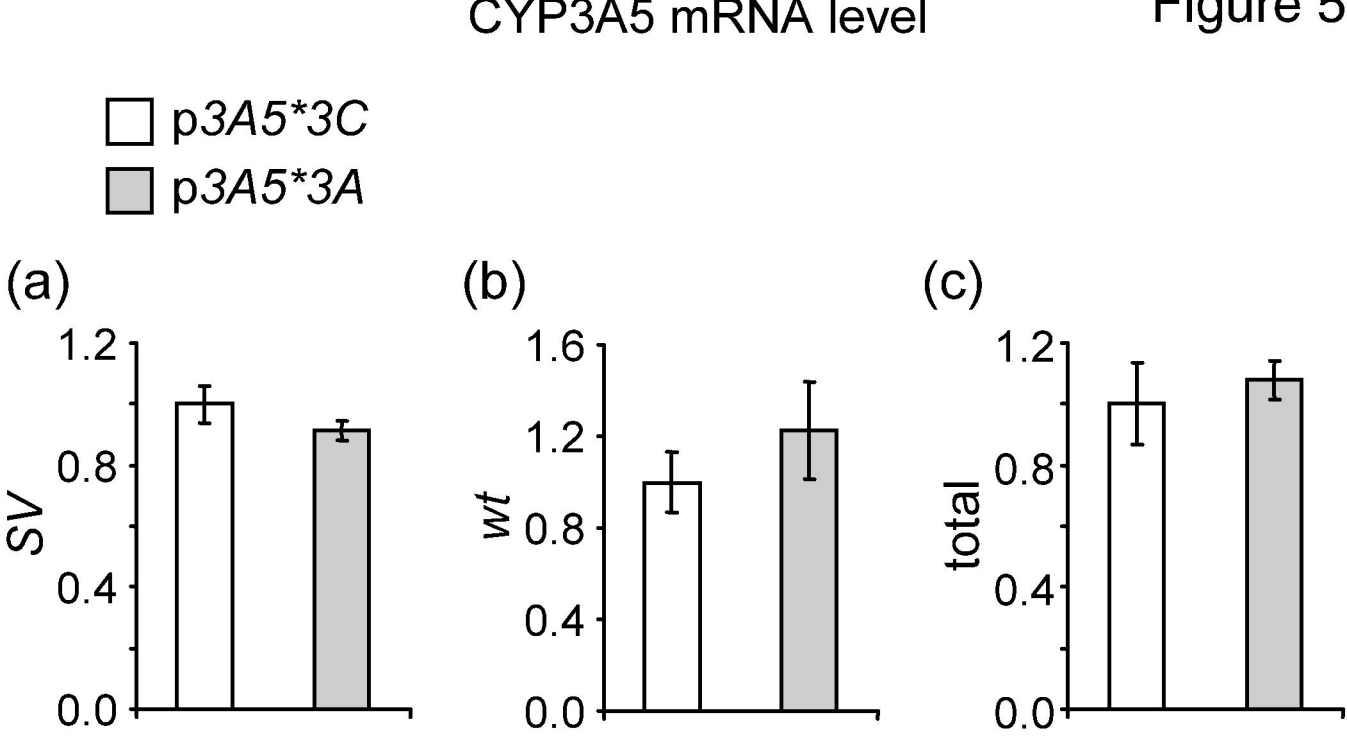

CYP3A5 mRNA level
p3A5*3C
p3A5*3A
(a)
SV
1.2
0.8
0.4
0.0
(b)
wt
1.6
1.2
0.8
0.4
0.0
(c)
total
1.2
0.8
0.4
0.0

Figure 6

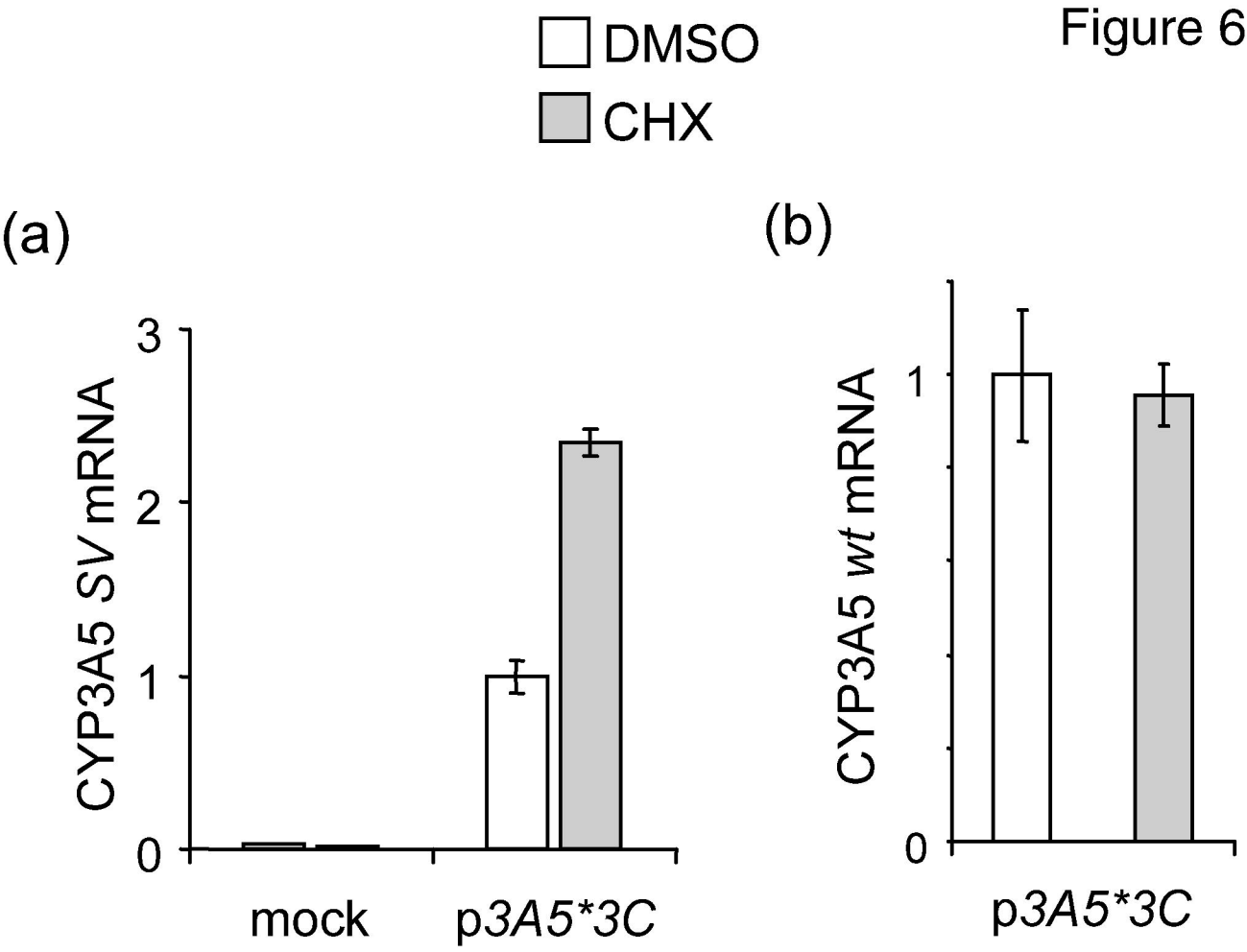

DMSO
CHX
(a)
CYP3A5 SV mRNA
3
2
1
0
mock
p3A5*3C
(b)
CYP3A5 wt mRNA
1
0
p3A5*3C

Figure 7

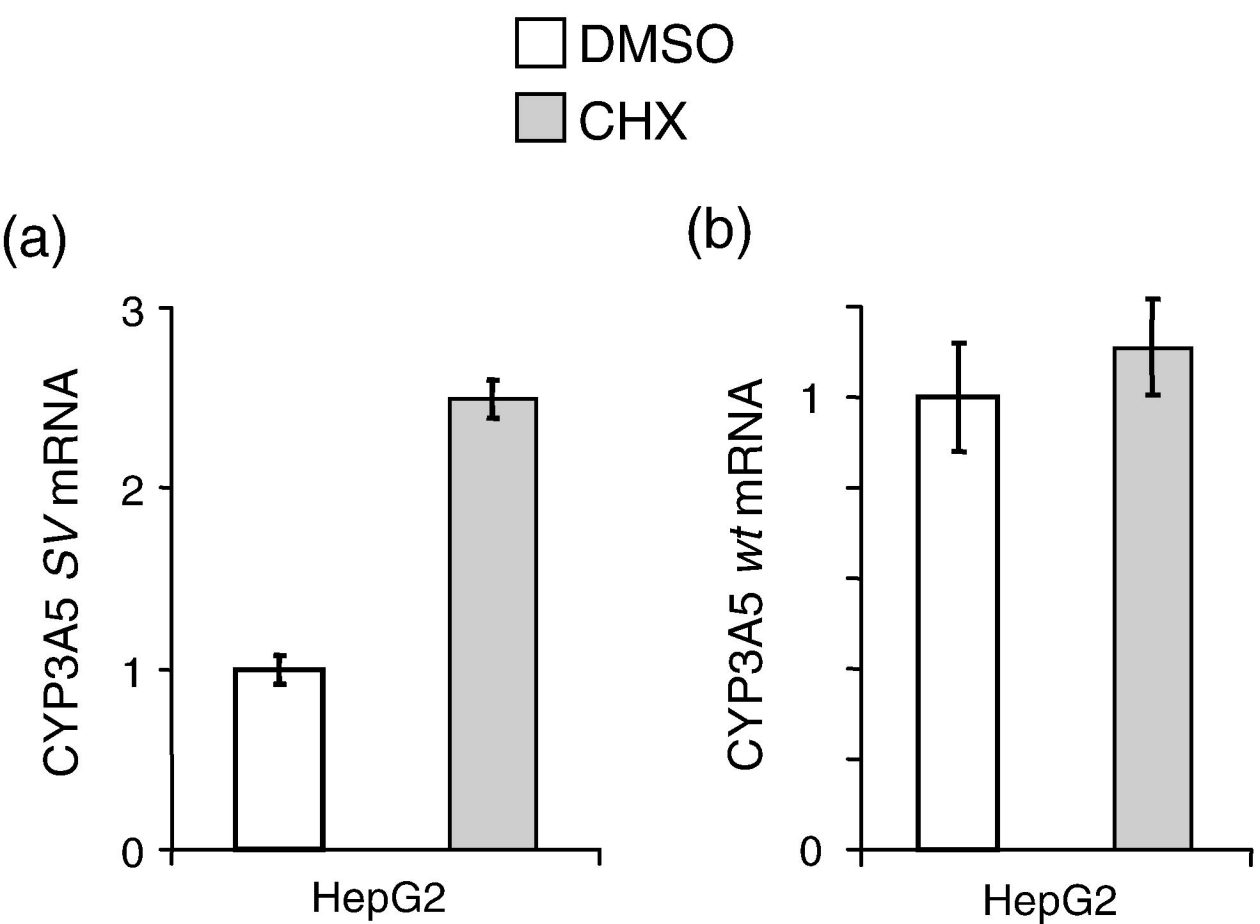


Figure 8

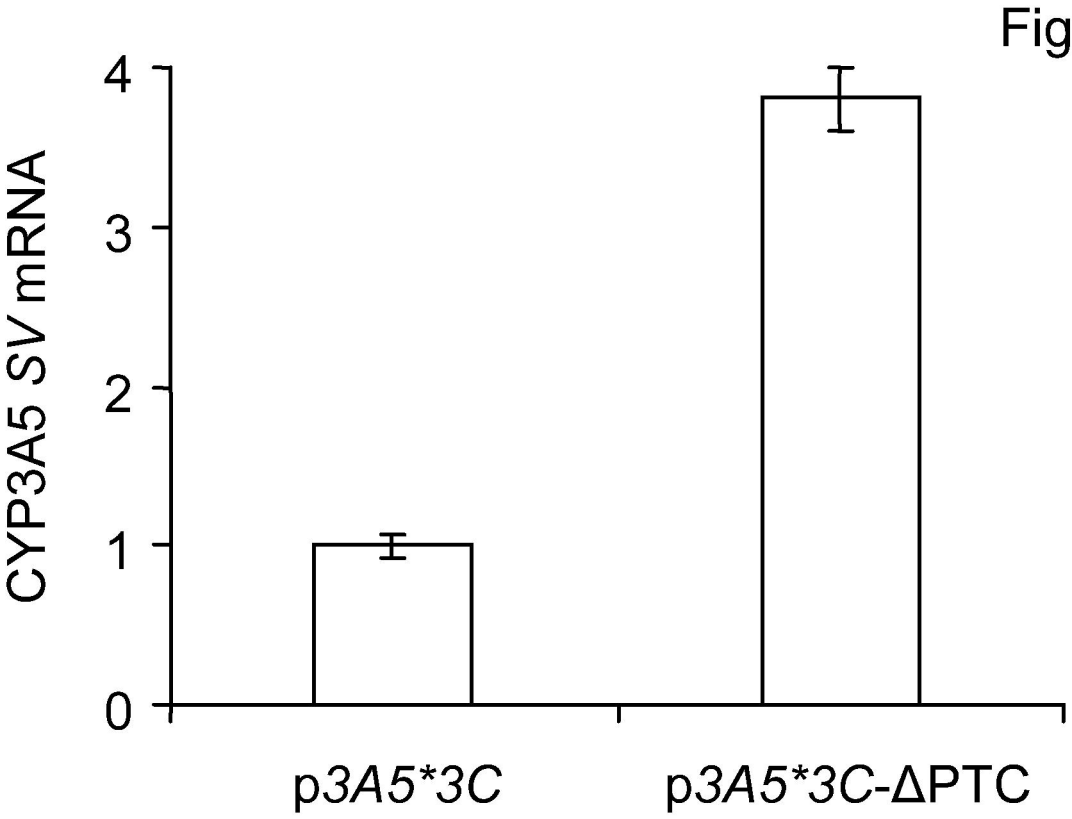
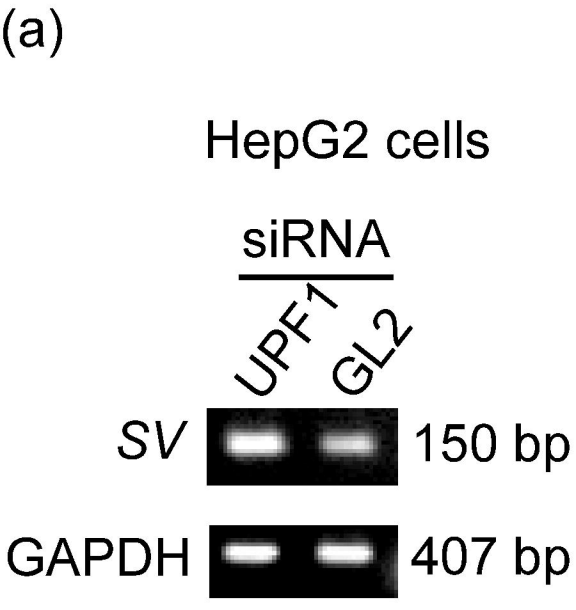
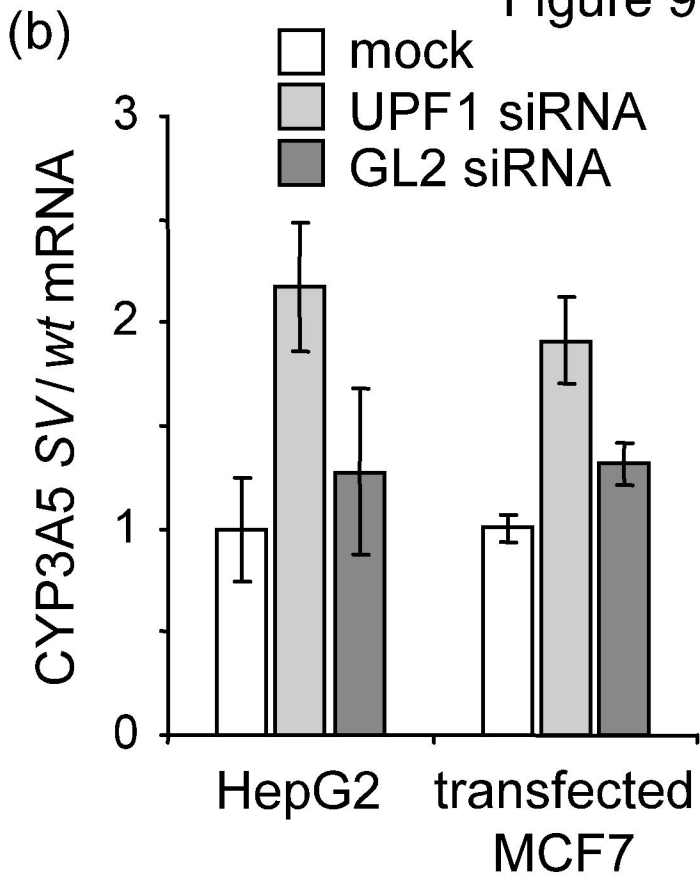


Figure 9


(a)
HepG2 cells
siRNA
UPF1
GL2
SV
150 bp
GAPDH
407 bp


(b)
mock
UPF1 siRNA
GL2 siRNA
CYP3A5 SV/wt mRNA
3
2
1
0
HepG2
transfected MCF7